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Time Saving Protein Binding Assay for the Simultaneous Determination of Guanosine 3′:5′-monophosphate (cGMP) and Adenosine 3′:5′-monophosphate (cAMP) in Human Urine

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Summary: A time-saving protein binding assay for the simultaneous determination of cGMP and cAMP has been adapted for human urine, using [^{3}H]cGMP, [^{14}C]cAMP, protein fractions from calf skeletal and lobster tail muscles and the phosphodiesterase inhibitor SQ 20.009. Recovery, accuracy, and precision are approximately at the 10% limit. Good specificity and no interference were observed with diluted urine samples (10 to 20 times).

Zeitsparende Proteinbindungsmethode für die simultane Bestimmung von Guanosin-3′:5′-monophosphat (cGMP) und Adenosin-3′:5′-monophosphat (cAMP) im menschlichen Urin

Zusammenfassung: Eine zeitsparende Proteinbindungsmethode für die simultane Bestimmung von cGMP und cAMP wurde für menschlichen Urin adaptiert; der Testansatz enthält [^{3}H]cGMP, [^{14}C]cAMP, Proteinfraktionen von Kalbskelettmuskel und Hummerschwanzmuskel sowie den Phosphodiesterasenhemmer SQ 20.009. Wiederauffindung, Genauigkeit, Präzision liegen um die 10%-Grenze. Mit 10–20fach verdünnten Urinproben werden eine gute Spezifität und keine Interferenz beobachtet.

Introduction

The determination of guanosine 3′:5′-monophosphate (cGMP) and adenosine 3′:5′-monophosphate (cAMP) is now performed routinely in many laboratories (1–3) and there is an increasing demand for time-saving simultaneous assays for both cyclic nucleotides (e. g. in urine), with respect to their use in clinical diagnosis. Recently two simultaneous protein binding assays have been described, one using binding protein fractions from bovine skeletal and lobster muscles (4), the other using extracts from beef adrenal cortex and the fat body of silkworm pupae (5). Both methods employ ^{3}H- and ^{32}P-labeled cyclic nucleotides in almost *equimolar* concentrations; the latter isotope, however, is very unstable on account of its short halflife value. Moreover, in the first procedure (4) the protein-nucleotide complexes were collected on cellulose ester filters, which requires a time consuming special filtration technique; the second procedure (5) used the precipitation technique with ammonium sulfate, which in turn requires time consuming washing and transfer processes for the protein-nucleotide complexes. Therefore in this paper we describe a simple simultaneous protein binding assay with stable ^{3}H- and ^{14}C-labeled cyclic nucleotides, using the time saving charcoal absorption technique, which requires only one centrifugation step. The assay has been adapted for the concentrations of cAMP and cGMP in human urine which exhibit a molar ratio of 5–10 to 1 (1–3). A preliminary report has been given in l. c. (6).

Materials and Methods

All labeled compounds came from Amersham-Buchler, Braunschweig, and NEN, Dreieichenhain, G.F.R., and were declared with a radio-chemical purity of 97–99%; [adenine-U-^{14}C]cAMP exhibits some chemical impurity of adenosine 5-monophosphate. All cyclic nucleotides and biochemicals were perchased from Boehringer, Mannheim, and the chemicals from E. Merck, Darmstadt, G.F.R. Norit A^3 was obtained from Norit Clydesdale, Glasgow, U. K.; it was suspended in double distilled water (1 g to 50 ml), centrifuged at 7800 *g* and the residue dried for further use. Bovine serum albumin was purchased from Behringwerke Marburg/L. and treated (7) to remove lipids and fatty acids. Urines of healthy persons were collected with theophylline during 24 h and immediately analysed.

Preparation and characterization of binding proteins

cAMP and cGMP binding protein fractions were prepared from calf skeletal muscle and from lobster tail muscle respectively; they were further purified by chromatography on DEAE-cellulose (Whatman DE 11) as described ((8) and (9), respectively). The isolated peak II- and peak I-fractions were dialysed

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Tab. 1. Effect of different buffer conditions on bound radioactivity and apparent dissociation constant of individual binding assays for cAMP.

	Buffer conditions						
	Tris-HCl buffer pH 7.4	Theophylline	SQ 20.009	2-Mercaptoethanol	$MgSO_4$	% of [^{3}H]cAMP bound	Apparent dissociation constant (cf. l. c. (16))
	(mmol/l)	(mmol/l)	(g/l)	(mmol/l)	(mmol/l)	(n = 5)	(nmol/l) (n = 2 – 4)
A (cf. l. c. (12))	55	4	–	3	–	30 ± 4	2.9 ± 0.8
B	120	5.3	–	4	–	24 ± 6	3.0 ± 0.9
C	120	–	0.3	–	–	26 ± 5	3.3 ± 0.7
D (cf. l. c. (11))	120	–	0.3	4	26.7	36 ± 5	2.6 ± 0.6

Total volume 0.15 ml, 0.25 pmol [^{3}H(G)]cAMP (1421 TBq/mol = 38.4 Ci/mmol)), 2.65 µg binding protein fraction from calf skeletal muscle. For further details see legend of fig. 3.

each at 4 °C against buffer (120 mmol/l Tris-HCl, pH 7.4), divided into 1 ml portions and stored at – 70 °C for several months without significant loss of binding activity. The protein fraction from calf skeletal muscle bound 0.2 pmol cAMP and < 0.001 pmol cGMP per µg protein (determined according to l. c. (10) using bovine serum albumin as standard); the protein fraction from lobster muscle bound 0.5 pmol cGMP and 0.045 pmol cAMP per mg protein after incubation with 14 nmol/l cyclic [8-^{3}H]cGMP (155 T Bq/mol (4.2 Ci/mmol)), or 10 nmol/l [^{3}H(G)]cAMP (710 T Bq/mol (19.2 Ci/mmol)), using the conditions and buffer D of table 1.

Results and Discussion

Conditions of the simultaneous protein binding assay

Conditions similar those described by *Dinnendahl* (11) for the cGMP binding assay were used. Therefore the conditions for the cAMP binding assay reported by *Brown* et al. (12) had to be changed with respect to the concentration of Tris-HCl buffer, which was increased (table 1). Furthermore, $MgSO_4$ was added and the concentration of 2-mercaptoethanol increased. Theophylline was replaced by SQ 20.009 (1-ethyl-4-(isopropylidene-hydrazino)-1 H-pyrazolol[3,-4-b] pyridine-5-carboxylic acid, a kind gift from Squibb, Princeton, N. J., U.S.A.), which is reported to inhibit the phosphodiesterase activity sixty-fold more than theophylline (13). Table 1 demonstrates that the altered conditions (especially SQ 20.009) do not influence significantly the amount of bound radioactivity nor the apparent dissociation constant of cAMP. Moreover, the conditions described with D in table 1 yield the same binding data for cGMP as are obtained with the *Dinnendahl* method (see below).

In order to evaluate optimal binding conditions, binding curves with increasing amounts of labeled cAMP or cGMP were established under the buffer conditions D of table 1 (figs. 1a, b). The saturation value for cAMP was established with 0.2 pmol/µg protein and for cGMP with 1.4 pmol/mg protein. Under these conditions, the apparent dissociation constant of the binding protein fraction from calf skeletal muscle was between 2–4 nmol/l cAMP (determined with [^{3}H(G)]cAMP (table 1) as well as with [adenine-U-^{14}C] cAMP (fig. 1b); and the protein from lobster muscle gave a value between 5–10 nmol/l cGMP (mean values of 4 experiments), confirming the values described by others (11, 14, 15).

Having combined both fractions of binding protein for the simultaneous assay, the apparent dissociation constant increased about 10 to 20 times with respect to cAMP binding and different charges of binding protein fractions. This obviously unspecific effect, also described for albumin (4, 14), was not altered by adding increasing amounts of cAMP. Under both conditions similar dose-binding curves were obtained (fig. 2). The apparent dissociation constant for cGMP was neither altered by the presence of skeletal muscle binding protein (cf. l. c. (4)), nor after the addition of 20 pmol or more cAMP (e. g. [adenine-U-^{14}C]cAMP). In both cases, the dose-binding curves showed linear log-log plots with slightly different slopes (fig. 2) indicating that [^{14}C]cAMP does not strongly interfere. The experimental binding data resemble the competitive inhibition data for an enzyme (which has nevertheless one and the same saturation value (cf. l. c. (16)). Moreover, under these conditions log-log plots of the dose-binding curves are linear for cAMP (range 200–1600 nmol/l) and cGMP (range 5–160 nmol/l) in the simultaneous binding assay (fig. 3).

The simultaneous protein binding assay for cGMP and cAMP described here has, however, one important disadvantage in comparison to those using ^{32}P-labeled cyclic nucleotides (4, 5): only 5% of [adenine-U-^{14}C] cAMP offered in the test is bound, so that the radioactive counts obtained are low, in contrast to those obtained with [^{3}H]cAMP and [^{3}H]cGMP, which are bound to an extent of more than 20%. The cause of the poor [^{14}C]cAMP binding remains unclear; contaminations of large amounts of cold cAMP or cGMP appear to be unlikely (fig. 2b, see above) and interferences with

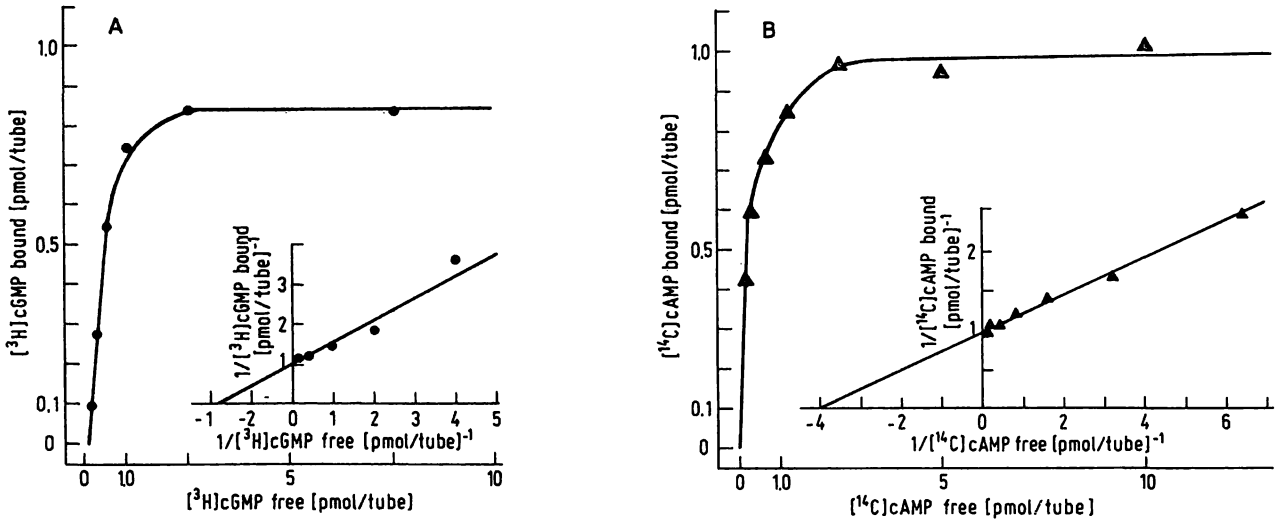

Fig. 1. Binding curves and double-reciprocal plots of cGMP and cAMP.
a) Increasing amounts of [8-^{3}H]cGMP (777 TBq/mol (21 Ci/mmol)) were incubated for 2 h at 4 °C together with 440 µg binding protein fraction from lobster muscle.
b) Increasing amounts of [adenine-U-^{14}C]cAMP (10.6 TBq/mol (287 mCi/mmol)) were incubated for 2 h at 4 °C together with 1.6 µg binding protein fraction from calf skeletal muscle. In both cases buffer conditions D of table 1 were used (total volume 0.1 ml). For further details see legend of fig. 3.

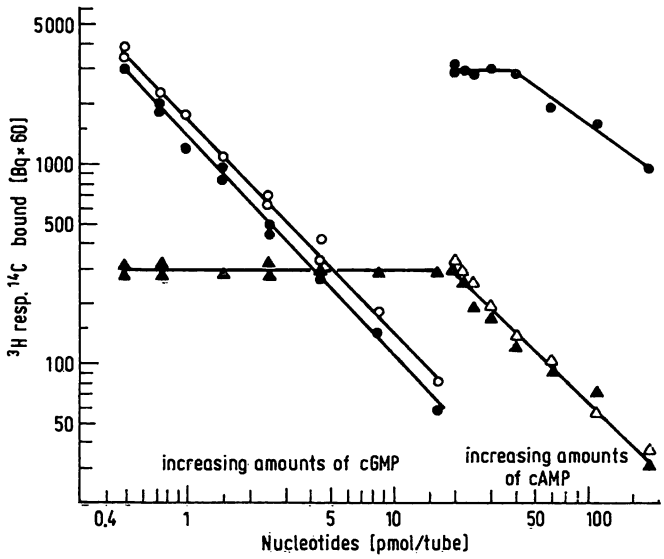

Fig. 2. Dose-binding curves for cAMP and cGMP on protein fractions from lobster muscle (G) and calf skeletal muscle (A).
a) o—o 5 µg protein A, 200 µg protein G, 0.5 pmol [ribose-5-^{3}H]cGMP (1.15 PBq/mol (31 Ci/mmol)) plus increasing amounts of cGMP as indicated on the cGMP scale.
b) •—• the same constituents as described under a) plus 20 pmol [adenine-U-^{14}C]cAMP (10.6 TBq/mol (287 mCi/mmol)); only increasing amounts of cGMP were added as indicated on the cGMP scale for [^{3}H]cGMP as well as on the cAMP scale for [^{14}C]cAMP, respectively.
c) △—△ 5 µg protein A, 200 µg protein G, 20 pmol [adenine-U-^{14}C]cAMP plus increasing amounts of cAMP as indicated on the cAMP scale.
d) ▲—▲ the same constituents as described under c) plus 0.5 pmol [ribose-5-^{3}H]cGMP. Only increasing amounts of cAMP were added as indicated on the cAMP scale for [^{14}C]cAMP and on the cGMP scale for [^{3}H]cGMP, respectively.
Total volume 0.1 ml; buffer conditions as described under D table 1. For further details, see legend of fig. 3.

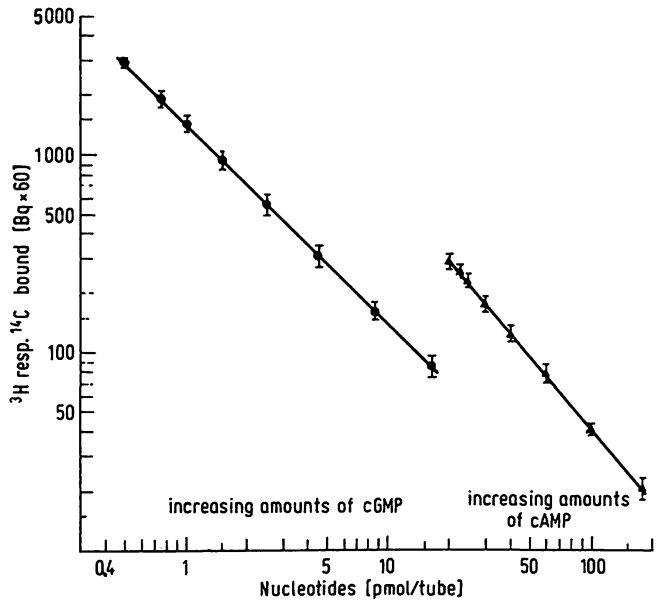

Fig. 3. Dose-binding curves for the simultaneous binding assay for cGMP and cAMP.
Mean values ± S. D. from 5 experiments. To a total volume of 100 µl were added 20 µl urine (diluted 10 to 20 times with buffer D, table 1), 0.5 pmol [ribose-5-^{3}H]cGMP (1.15 PBq/mol (31 Ci/mmol)), 20 pmol [adenine-U-^{14}C]cAMP (10.6 TBq/mol (287 mCi/mmol)) and buffer (conditions D of table 1); after mixing, the reaction was started with 5 µg binding protein fraction from calf skeletal muscle plus 200 µg binding protein fraction from lobster muscle and incubated for 2 h at 4 °C; blank without proteins, sample volume 20 µl. Then 50 µl charcoal suspension (30 g/l charcoal, 10 g/l albumin in buffer D of table 1) were added to all samples simultaneously which were centrifuged at 2000 *g* for 20 min at 4 °C. 100 µl clear supernatant were mixed with 400 µl bidist. water in 10 ml Unisolve I (Zinsser, Frankfurt/M., G.F.R.) and counted in a Tri-Carb model 3380 with automatic unit model 544 (Packard Instruments, Frankfurt/M., G.F.R.) with a counting error of less than 5%.

other nucleosides or nucleotides occur only with higher concentrations (e. g. 1 mmol/l adenosine-5-monophosphate inhibited < 5% [^{3}H]cAMP binding, for further details see below).

Accuracy

The simultaneous recovery (n = 45) of 20 pmol cAMP and 2 pmol cGMP in urine samples exhibits values between 89% and 111% and 92% and 108%, respectively. Comparing the results of two urines measured by individual binding assays for cGMP (cf. l. c. (11)): 1000; 330 nmol/l) and for cAMP (cf. l. c. (12)): 6.70; 2.90 µmol/l) with those determined by the simultaneous binding assay (cGMP: 1050; 340 nmol/l; cAMP: 6.30; 2.80 µmol/l) the values are in accordance below the 10% limit. Furthermore, the data for two urines obtained in the presence of both binding proteins correspond well (below the 10% limit) with those measured by the simultaneous binding assay: for cAMP: 6.26 (8.51) and 6.88 (8.23) µmol/l; for cGMP: 585 (948) and 594 (888) nmol/l.

Precision

The coefficient of variation from day to day (10 different experiments) ranges between 8 to 10% for cGMP and between 5 to 12% for cAMP, when both nucleotides were estimated simultaneously in molar cGMP to cAMP ratios of 1 to 2.5 and 1 to 40. Similar coefficients of variation are exhibited by the recovery experiments with a molar ratio of cyclic nucleotides of 1 to 20 as demonstrated above. The coefficient of variation within series shows somewhat lower values: 5 to 8% and 5 to 9%, respectively.

Specificity

Although, in the simultaneous assay, cAMP is bound by two different binding protein fractions, log-log plots of the dose-binding curves are linear, and they do not show any disturbance with increasing concentrations of cGMP (5–160 nmol/l, figs. 2, 3). 10 times higher concentrations of cGMP begin to displace cAMP from skeletal muscle binding protein. cGMP is only bound by the protein fraction from lobster muscle; however, cAMP concentrations > 400 nmol/l begin to displace labeled cGMP, thus exhibiting a 50 to 100 times higher value for the apparent dissociation constant. Therefore urine samples have to be diluted 10 to 20 times with buffer D (table 1); otherwise cGMP values must be corrected (e. g.: 500 nmol/l cAMP displace ~ 1.0 nmol/l cGMP, 800 ~ 2.6, 1600 ~ 6.8; conditions of fig. 2). Nevertheless, in the diluted urine samples the cGMP concentration appears to be high enough for its exact measurement.

Interference

Interference by corresponding purine bases, nucleosides or nucleotides (e. g. adenosine-(guanosine-)mono-, di-, triphosphate) occurs with 10 to 1000 times higher concentrations than are to be expected in the test (cf. l. c. (11, 12, 15, 17)). To exclude a chemical interference, individual blanks of the diluted urine samples are used, and generally they have proved to be low.

Cyclic nucleotide levels in 12 human urines were measured by the simultaneous binding assay (conditions see fig. 3) and related to the urine volume excreted during 24 h or to 1 mol creatinine (determined with the Technicon Autoanalyzer). In 5 healthy men (44 ± 9 years old) the value for cAMP was 6.73 ± 2.12 µmol/24 h (0.169 ± 0.043 mmol/mol creatinine) and for cGMP 649 ± 179 nmol/24 h (16.3 ± 3.2 µmol/mol creatinine), in 7 healthy women (38 ± 12 years old) 5.51 ± 1.03 (0.191 ± 0.047) and 648 ± 219 (22.6 ± 6.2). The data correspond well with those determined by other procedures (cf. l. c. (1, 3, 14)).

Summing up, although the counting (and binding) yields are low with respect to [^{14}C]cAMP, the precision, accuracy and recovery of the simple simultaneous protein binding assay adapted for human urine are quite satisfactory for cAMP and cGMP, and they are comparable to those of other individual or simultaneous techniques already described (1, 3, 14; 4). The time-saving assay (up to 50 samples may be analysed in triplicate per day) appears to considerably lower the cost, compared with commercially available individual protein binding assays.

Note added to the proof:
Using other batches of [U-^{14}C-adenine]cAMP and binding protein fractions of lobster tail muscles 9 to 15% of [U-^{14}C-adenine]cAMP offered in the test are bound, so that the radioactive counts obtained are at least two times higher than described, indicating now a better sensitivity of the test.

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